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Traditional and molecular analyses of the genes encoding pneumococcal polysaccharide capsule production: cloning Avery's transforming principle

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The pneumococcus constitutes one of the most important human pathogens and is among the most thoroughly studied of all bacteria. Virulence in humans is conferred primarily by the presence of an antiphagocytic extracellular polysaccharide capsule. Perhaps the greatest biological discovery of the twentieth century can be directly linked to investigations of the pneumococcal capsule. Specifically, following earlier work on transformation by Fred Griffith and others, Oswald Avery and colleagues showed that DNA, and not some other cellular component was responsible for the transformation of pneumococci from an unencapsulated to an encapsulated state. This finding led to research demonstrating that genes responsible for encapsulation are closely linked. Almost twenty years elapsed prior to the initiation of work designed to delineate the molecular architecture of the encapsulation genes. The rate of progress in this area is increasing rapidly at present and offers great promise for the complete analysis of Avery's transforming principle.

INTRODUCTION

The pneumococcus and the foundations of modern molecular biology

Since its nearly simultaneous identification in 1881 as a human pathogen on two continents by Pasteur in Europe and by Sternberg in North America, *Streptococcus pneumoniae*, the pneumococcus, has been the subject of intense scrutiny by both clinicians and basic researchers. Because of this keen interest by a variety of scientists, an impressive number of breakthrough discoveries in biomedicine have occurred using this important bacterial pathogen of humans [1]. Among these are several related in various ways to its extracellular polysaccharide capsule, the presence of which has clearly been demonstrated to be required for virulence [2]. This covering is composed of heterogeneous mixtures of five or six carbon monosaccharides linked into long linear or branched polysaccharide polymers. In fact, the discovery that genes are made of DNA was the result of work done to transfer the ability to make a capsule of a given serotype from one pneumococcus to another [3].

In this paper we will outline a number of early studies of the pneumococcal capsule, including those that led directly to the identification of the transforming principle. We will proceed to a discussion of some classic papers which demonstrated that the structural genes for capsule production are linked. We will then review a number of ongoing studies that illustrate the power of modern molecular biological tools in the elucidation of the actual nucleotide sequences of capsulation genes. This review will conclude with a summary of both historical and recent advances in the genetics of capsule production in the pneumococcus.

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EARLY STUDIES

Oswald Avery and capsular polysaccharides

Almost from its discovery, the pneumococcus has been recognized as a very important cause of pneumonia. This was certainly true in 1913 when a young clinician named Oswald T. Avery took on the task of understanding how this pathogen produced disease [4]. Within a short time, Avery and his more senior associate Alphonse Dochez made what was for its time an astounding discovery, namely, that the principal antigen of the pneumococcus that was responsible for eliciting an immune response was not a protein, but rather, a composite sugar, or polysaccharide [5]. Over the next several years, a number of investigators demonstrated that these polysaccharides were immunogenic, and that preparations of isolated capsular material were capable of producing a protective immune response (see reference 6 for a review). Michael Heidelberger, another Avery associate, described the capsule as follows: '. . .disposed at its [the pneumococcus] periphery [is] a highly reactive substance upon which type specificity depends' [7]. It is now known that there are at least 85 immunochemically distinct polysaccharide types (serotypes) comprising the capsule of this organism [1].

Griffith's R and S: the transforming substance

At the same time, across the Atlantic Ocean a British Public Health Service physician–researcher was conducting his own investigations of the pneumococcus. Fred Griffith was interested both in the distribution of serotypes causing lobar pneumonia and in the phenomenon of lack of mouse virulence among the so-called rough pneumococci (since it was well-known even at that time that mice are extremely susceptible to infection by pneumococci). He employed an improved version of the rough (R) and smooth (S) designations applied previously by Arkwright [8] when referring to these spontaneously unencapsulated mouse-avirulent, or R, organisms. Griffith had observed on several occasions that sputum from persons suffering from lobar pneumonia contained multiple serotypes of pneumococci. He believed that it was equally likely to be the result of the transfer of information required for the production of different capsular types or that multiple episodes of acquisition of pneumococcal strains had occurred. He therefore undertook to determine whether there was a particle or some other type of carrier of information that could convert avirulent R forms of the bacterium to highly virulent S forms. To accomplish this he injected (along with appropriate controls) heat-killed S pneumococci and live R pneumococci. The result was that Griffith was able to isolate live, smooth bacteria from moribund, and in some cases, dead mice. The obvious implication waas that something had indeed been transferred from the heat-killed pneumococci to the live organisms. Griffith had little or no interest in the biochemical identity of this substance [9]; nonetheless, his results were rapidly reproduced. In retrospect, it is at least theoretically possible that no information was passed, since one can imagine that the mouse possesses the intrinsic capacity to degrade extracellular DNA. This hypothesis requires an alternate mechanism to explain the production of an encapsulated organism from Griffith's rough pneumococcus; such a mechanism will be offered later in this paper.

Reproducibility and extension of Griffith's studies

The work of Griffith was quickly digested and repeated by Neufeld and Levinthal in Germany [10], largely because Neufeld had just visited Griffith in London and had observed the techniques involved [11]. In short order, Dawson and colleagues in New York City showed that the transformation reaction could take place in the test tube [12–15]. Alloway was able to extend the findings of these researchers to include a demonstration that the mysterious transforming particle was contained within water-soluble, filterable, cell-free extracts of the bacterium [16, 17].

DNA as the biochemical identity of the gene

By the early 1930s, based on the investigations just mentioned, Oswald Avery was convinced that it should be possible to uncover the biochemical identity of the particle inducing transformation of pneumococcal serotypes. He and a number of colleagues set out to analyse systematically the various components of the bacterium, including proteins, carbohydrates and nucleic acids in an attempt to accomplish this goal. By the late 1930s, it was clear that the transforming principle, as they then called it, consisted mostly, or perhaps entirely, of deoxyribonucleic acid. Recently, Maclyn McCarty suggested that the first written mention by Avery that the transforming principle consisted of DNA occurred in the 1935 annual report by Avery to the Rockefeller Board of Governors [18]. Even so, the prevailing view within the genetics community

at that time was that genes were likely to be composed of protein, in whole or in part, in order to allow for the encoding of complex information. This prompted Avery and his colleagues to perform even more experiments designed to prove unequivocally that DNA was the molecule responsible for transformation. It was therefore not until some years later, in 1944, that Avery, MacLeod, and McCarty published their landmark paper in the Journal of Experimental Medicine [3] demonstrating that DNA was the carrier of genetic information. The implications of this finding were at least two-fold. First, the gene, the individual unit of heredity, must encode all of its information through various combinations or sequences of the four constituent nucleotides of DNA, since only this molecule was found to be capable of inducing the changes brought about by transformation. The second major finding was that bacteria can actively acquire new genetic information from the surrounding milieu, in a process now known as natural transformation.

The hypothesis of DNA as the biochemical identity of the gene was further validated by studies reported by McCarty in 1946 and by Rollin Hotchkiss in 1951. McCarty showed that purified desoxyribonuclease, an enzyme that specifically hydrolysed DNA, could completely abolish the transforming activity of DNA [19]. Hotchkiss demonstrated that genes other than those encoding the production of extracellular capsules (i.e. one or more genes conferring resistance to penicillin) could be transformed from strain to strain via transformation [20].

CLASSICAL LINKAGE STUDIES AND THE PNEUMOCOCCUS

Early studies

In 1949, even before the work of Hotchkiss became known, Harriet Ephrussi-Taylor suggested that capsulation genes might exist as a cluster, or cassette, of closely linked sequences [20]. Further, she hypothesized that this cluster might occupy the same chromosomal location in all pneumococci. Transformation of a given strain should then result in a pneumococcus of altered capsule type, but with an otherwise unchanged genotype. This concept was supported with data from her own studies [21, 22], as well as those of Austrian and Bernheimer [23, 24] and Ravin [25, 26]. Work by others illustrated that a number of pneumococcal serotypes could be transformed to a changed polysaccharide capsule specificity [15, 27].

Binary capsulation and linkage

Research conducted by Austrian and Bernheimer and coworkers beginning in the late 1950s and continuing through the 1960s and early 1970s shed additional light on the concept of a cluster of genes capable of altering capsular type. They stated '. . it appears that, when transformation occurs with acquisition of new biosynthetic capabilities and loss of ones possessed previously by the cell, a reaction analogous to 'crossing over' has taken place between the two capsular genomes . . .' [23]. Later, in an elegant series of classical genetic manipulations, Bernheimer and colleagues demonstrated that mutations within genes for enzymes of common biosynthetic pathways could be complemented via transformation using DNA from pneumococci of heterologous capsular specificity. Moreover, in some cases they produced transformed strains expressing two polysaccharides, the so-called binary strains. This was probably due either to limited homology of the ends of the transforming DNA fragment carrying the genetic information for the production of the second capsular polysaccharide [29], or to the result of a single Campbell-type cross-over event in which all the genes encoding the production of the second polysaccharide type were added to one end of the region encompassing the genes encoding the production of the original serotype [30, 31].

MOLECULAR APPROACHES TO THE STUDY OF CAPSULATION GENES

The transforming principle as capsulation sequence

As has been discussed above, the genes contained within the transforming principle identified by Avery and colleagues included a cluster involved in the production of the serotype 3 capsule. At the time of the publication of the famous 1944 paper [3], this group was not aware of exactly how many genes were transferred, but following the appearance of work by both Hotchkiss [20] and Ephrussi-Taylor [22, 23], it was obvious that the information being exchanged was variable. Hotchkiss convinced sceptics of the general nature of the ability of DNA to carry genetic information by transforming pneumococci from penicillin susceptibility to penicillin resistance; Ephrussi-Taylor transformed pneumococci from one serotype to another, illustrating that the transforming princi-

ple could be of different specificities. Surprisingly, it has only been within the past few years that a number of groups have begun to unravel the molecular structure of these genes. Technical difficulties have hindered the study of pneumococcal DNA, but recent improvements promise rapidly to increase knowledge of the pneumococcal genome. Considerable progress has now been made in uncovering the molecular architecture of pneumococcal capsule production, as summarized in the following section.

Application of modern molecular techniques to capsulation sequences

In 1990, we showed that insertion of the conjugative Gram-positive transposon Tn*916* was sufficient to render *S. pneumoniae* serotype 3 unencapsulated (Figure 1) and avirulent [2] as judged by intraperitoneal inoculation of as many as 1×10^8 c.f.u. of the mutant organism into Swiss outbred mice. The original mutant strain, DW3.7, was found to contain four copies of the transposable element within its genome; since our goal was to examine DNA essential for serotype 3 encapsulation, we isolated the insertion mutation responsible for unencapsulation using transformation into a new serotype 3 background different from the parent strain. While both DW3.7 and DW3.8 were avirulent in mice, we observed that on some occasions, one or more mice within a group challenged with the mutant strain DW3.7 became moribund [2]. Re-isolation of pneumococci from blood did not produce wild-type revertant mucoid serotype 3 penumococci, but rather, a pneumococcus with an apparently rough colony phenotype. Subsequent detailed analysis has led to the conclusion that the re-isolated organism is not unencapsulated, but is in fact encapsulated by a serotype different from that of the parent, and is therefore no longer mucoid. Based on blinded serotyping done by laboratories in the USA, The Netherlands, and Denmark, we believe this isolate to be serotype 4. Moreover, protection of Swiss outbred mice from challenge by this organism is conferred by vaccination with heat-killed serotype 4 organisms (of either the American Type Culture Collection [ATCC] type strain or the isolate itself), with pneumococcal polysaccharide type 4 (obtained from the ATCC), or with the 23-valent pneumococcal vaccine (obtained from Merck, Sharp, and Dohme, West Point, PA). No protection from challenge could be obtained by vaccination with the parent serotype 3 strain, rough mutant DW3.8, or isolated purified pneumococcal cell wall. We hypothesize that it is at least theoretically possible that more than one cluster of capsulation genes exists within the chromosome of this strain, and that mouse passage somehow results in expresssion of the second, previously silent group of capsule genes. Thus, an alternate explanation for the original finding of Griffith might be that a second, unexpressed locus of capsulation genes was present in the transposon mutant R strain, and that upregulation of its expresssion occurred in vivo.

Detailed molecular biological analysis of a 2.8 kb fragment of DNA surrounding the transposon insertion in mutant strain DW3.8 has revealed several interesting features suggestive of a role for this region of DNA in the regulation of serotype 3 capsule production [32]. The first is that this insertion mutation is only approximately 300 nucleotides 3' of the transcriptional terminator of *lytA*, the pneumococcal autolysin gene; it has been hypothesized that the cell wall lytic enzyme encoded by this gene is involved in the control of cell wall synthesis. The second feature of this sequence is that unencapsulated mutants described by Sanchez-Puelles et al [33] and by Diaz, Lopez and Garcia [34] have a deletion in this region relative to *lytA*. The third is that the transposon insertion is 5' of what may be a small transcribed open reading frame, which is itself just 5' of a copy of the newly described BOX repeat element of pneumococcus [25]. It will be of great interest to examine further the role of these elements in the regulation of pneumococcal capsule production given their proximity to a number of important pneumococcal genes (e.g. genes encoding autolysin, the toxin pneumolysin, and one of the penicillin-binding proteins, to name only some).

As mentioned above, four separate groups of researchers have begun to examine the structural genes of three serotypes of the pneumococcus. Nuijten and coworkers [36], using the cloned *S. agalactiae* serotype III capsulation gene *cpsD* obtained from Rubens et al [37] as a probe, have cloned and sequenced roughly 2000 nucleotides of the 5' end of a gene from *S. pneumoniae* serotype 14; this gene appears to contain a high degree of homology with the galactosyl transferase gene (*cpsD*) of *S. agalactiae* serotype III. Preliminary data from Guidolin and colleagues in Australia [38] has revealed a very high level of homology within a 7.5 kb fragment of *S. pneumoniae* serotype 19F DNA to the same *S. agalactiae* serotype III cloned capsulation sequence of Rubens et al [37]. Work is ongoing

in both groups to identify and sequence longer contiguous regions of the capsulation sequences. In addition, two laboratories have made substantial progress in analysing the genes encoding the serotype 3 polysaccharide capsule. Garcia et al [39] have identified the uridine diphosphate-glucose dehydrogenase (UDP-GDH) gene of *S. pneumoniae*, as well as an approximately 10 kb *Eco*RI fragment involved in the production of the serotype 3 pneumococcal polysaccharide [40]. They have localized this restriction fragment to a 290 kb *Sma*I restriction fragment of the pneumococcal chromosome. Kelly, Dillard and Yother [41] have shown that the genes encoding this capsular serotype are linked and contained within a defined chromosomal location. Further, they have shown that the DNA sequence at one end of the locus is common to a number of serotypes, while that at the opposite end is likely to be serotype-specific [42]. Based on sequencing thus far reported, the sequence obtained by Dillard and Yother again includes UDP-GDH, as well as glucose-1-phosphate uridylyltransferase, and a serotype 3 polysaccharide synthase [43].

Historical perspective

Sixty-six years ago, Fred Griffith set out to understand how humans could acquire multiple serotypes of pneumococci during the course of lobar pneumonia. The major finding of his work was that heat-killed virulent pneumococci could pass the information for the encapsulated, or smooth, phenotype to otherwise avirulent strains of this organism. The search for the biochemical identity of this factor lasted 16 years, and occurred in stages, as described in this paper (and as shown in Figure 2). It concluded fifty years ago, when Avery, MacLeod and McCarty published a breakthrough paper [3] using the pneumococcus as the centrepiece of their experimental system, and transfer of

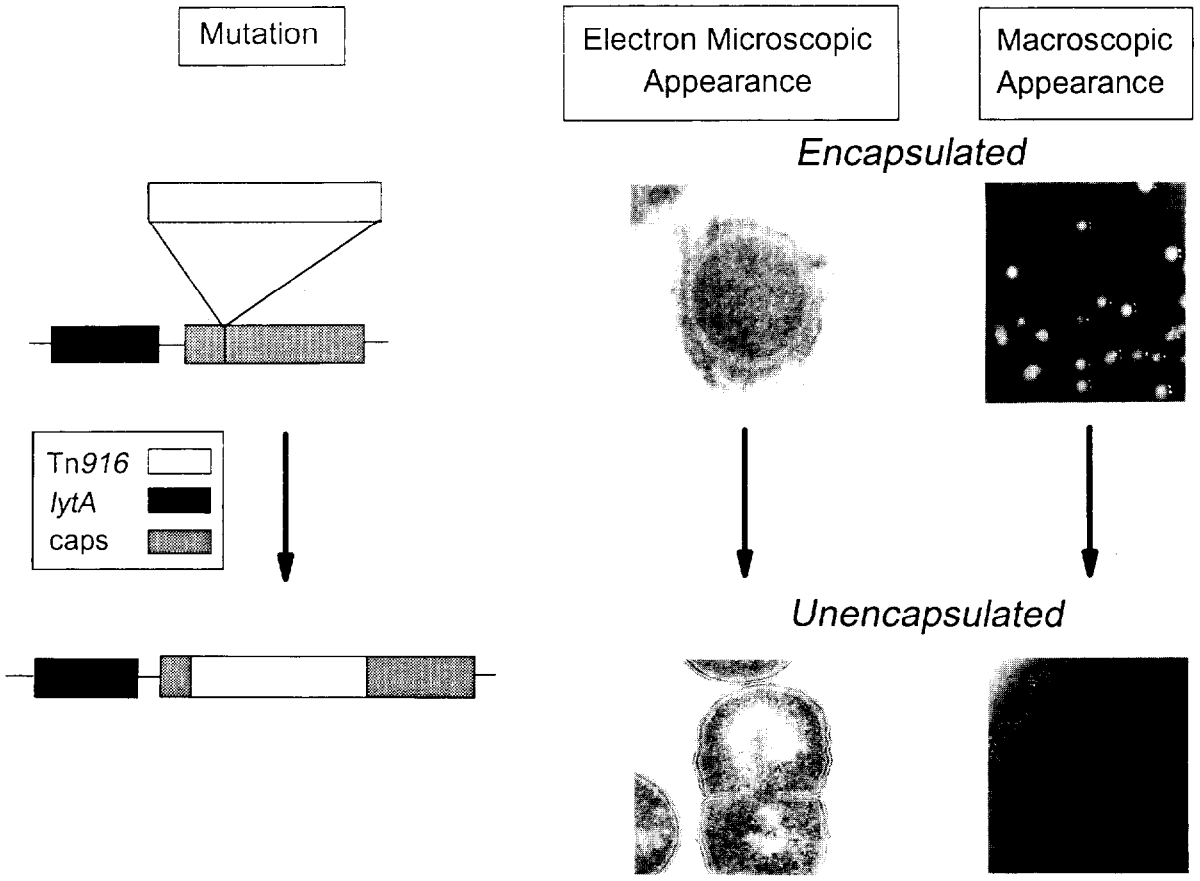

Fig. 1. (from left to right) Transposon Tn*916* is shown diagramatically inserting into a putative capsulation-required DNA sequence just downstream from *lytA*, the gene encoding the pneumococcal autolysin; next, the electron microscopic appearance of the parental wild-type serotype 3 strain and of the transposon insertion mutant strain DW3.8 are shown (same magnification); lastly, the macroscopic colony morphologies of these same two strains are illustrated (same magnification).

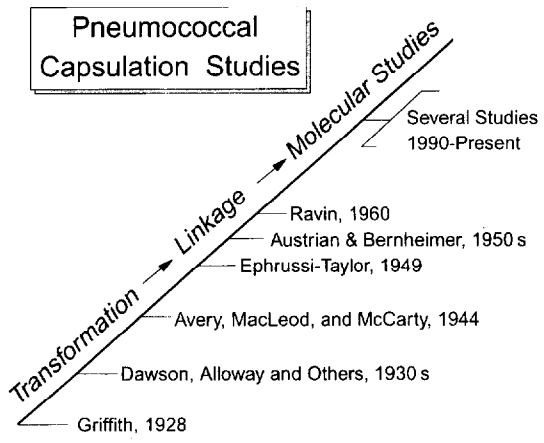

Fig. 2. A chronological illustration of milestones in the history of the study of the transforming principle.

the genes responsible for serotype 3 capsule production (and therefore the mucoid serotype 3 phenotype) as their indicator of success. In retrospect, this single piece of research signaled the birth of an era (as has recently been discussed by Joshua Lederberg [44]). For Avery, it represented the capstone to a brilliant lifetime of study devoted to the pneumococcus; for MacLeod, an outstanding beginning to a distinguished career that would see him complete one of the earliest and most important proofs of the efficacy of purified pneumococcal polysaccharide vaccines; and for McCarty, the first of many important works concerning the streptococci. While none of these scientists could have predicted the extent to which their paper would have an impact on the future of the biological sciences, McCarty has recently said that '. . . it didn't take long [when working on the transforming principle in the Avery laboratory] to appreciate the genetic implications of what we were doing . . .' [18]. During the 1950s and 1960s, bacterial geneticists were able to prove that those genes involved in capsule production were in fact linked and could be transformed as a unit. Given the enormous importance of the discovery by the Avery group however, it is indeed ironic that a half-century has passed and only now is the molecular architecture of the transforming principle being delineated, as discussed above. We believe it is not unreasonable to speculate that with the complete sequencing and analysis of the capsulation genes from several serotypes of *S. pneumoniae* imminent, it may soon be possible to control expression of these genes, and therefore virulence, of this important human pathogen.

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